C-terminal region of activation-induced cytidine deaminase (AID) is required for efficient class switch recombination and gene conversion

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Activation-induced cytidine deaminase (AID) introduces single-strand breaks (SSBs) to initiate class switch recombination (CSR), gene conversion (GC), and somatic hypermutation (SHM). CSR is mediated by double-strand breaks (DSBs) at donor and acceptor switch (S) regions, followed by pairing of DSB ends in two S regions and their joining. Because AID mutations at its C-terminal region drastically impair CSR but retain its DNA cleavage and SHM activity, the C-terminal region of AID likely is required for the recombination step after the DNA cleavage. To test this hypothesis, we analyzed the recombination junctions generated by AID C-terminal mutants and found that 0- to 3-bp microhomology junctions are relatively less abundant, possibly reflecting the defects of the classical nonhomologous end joining (C-NHEJ). Consistently, the accumulation of C-NHEJ factors such as Ku80 and XRCC4 was decreased at the cleaved S region. In contrast, an SSB-binding protein, poly (ADP)-ribose polymerase1, was recruited more abundantly, suggesting a defect in conversion from SSB to DSB. In addition, recruitment of critical DNA synapse factors such as 53BP1, DNA PKcs, and UNG at the S region was reduced during CSR. Furthermore, the chromosome conformation capture assay revealed that DNA synapse formation is impaired drastically in the AID C-terminal mutants. Interestingly, these mutants showed relative reduction in GC compared with SHM in chicken DT40 cells. Collectively, our data indicate that the C-terminal region of AID is required for efficient generation of DSB in CSR and GC and thus for the subsequent pairing of cleaved DNA ends during recombination in CSR.

DNA repair | CSR synapse

Activation-induced cytidine deaminase (AID) is essential for three different genetic events: class switch recombination (CSR), gene conversion (GC), and somatic hypermutation (SHM), which contribute to Ig gene diversification (1–5). Although AID generates single-strand breaks (SSBs) in the Ig genes, subsequent repair steps for CSR and GC are similar to each other but are distinct from SHM in their mechanistic properties, i.e, in (*i*) generation of the double-strand breaks (DSBs), (*ii*) recombination, and (*iii*) the requirement for uracil-DNA-glycosylase (UNG) for the pairing of the DSB ends (6–10). Despite the similarities between GC and CSR, their repair mechanisms have distinct features: CSR recombination requires nonhomologous end joining (NHEJ), and GC depends on homologous recombination (HR). During CSR, DSB ends normally are joined by classical NHEJ (C-NHEJ), which requires specific repair proteins such as Ku80, XRCC4, or DNA ligase IV (11, 12). In the absence of C-NHEJ, a back-up end-joining pathway called "alternative end joining" (A-EJ), which is reported to be slower and also more error prone than C-NHEJ, joins the broken DSBs ends (13). On the other hand, HR, the most common form of homology-directed repair, requires long sequence homology between donor and acceptor DNA to complete the recombination step by recruiting a distinct set of repair proteins such as RAD54, RAD52, and RAD51 to the break sites (14, 15).

Various studies on AID mutations in the N-terminal or C-terminal regions (4, 8, 9, 16–19) have shown that N-terminal AID mutants are compromised for CSR and are defective in SHM, indicating that the N-terminal region of AID is required for DNA cleavage (9, 16, 19). On the other hand, the C-terminal region of AID, which contains a nuclear-export signal and is responsible for AID's shuttling activity between the nucleus and cytoplasm, is required for CSR-specific activity but not for DNA cleavage activity and SHM (8, 16). Among the series of AID C-terminal mutants examined, two mutants show characteristic features: P20, which has an insertion of 34 amino acids at residue 182 and normal nuclear-cytoplasmic shuttling activity, and JP8Bdel, which has a 16-amino acid truncation at residue 183, accumulates in the nucleus, and shows higher DNA break activity at the donor switch (S) region (16, 17). Although several reports suggest that the C-terminal region of AID is involved in protein stability (20, 21), C-terminal mutants of AID stabilized by fusing the hormone-binding domain of estrogen receptor (ER) also show similar CSR-defective phenotypes (8). Taken together, these data suggest that the DNA cleavage activity and CSR-specific activity depend on different regions of AID (8, 19). In addition, the C-terminal region of AID is essential for the interaction of AID with poly $(A)^+$ RNA via a specific cofactor (22). Because CSR requires de novo protein synthesis, we proposed that after DNA cleavage the C-terminal region of AID may be involved in the regulation of the recombination step through generation of a new protein (8, 16, 22).

Significance

Activation-induced cytidine deaminase (AID) initiates class switch recombination (CSR) by inducing Ig locus-specific single-strand breaks (SSBs). AID C-terminal mutants (C-mt) generate SSBs efficiently but fail to support CSR. We found that residual CSR junctions in AID C-mt were repaired predominantly by alternative end-joining repair and that the recruitment of classical nonhomologous end-joining factors such as Ku80 to the S region was reduced consistently. Conversely, the accumulation of poly (ADP)-ribose polymerase1 was observed in the AID C-mt JP8Bdel. AID C-mt also showed a relative reduction in gene conversion (GC). Moreover, AID C-mt did not support synapse formation in the donor switch regions, indicating that the C-terminal region of AID is essential for efficient generation of double-strand breaks in CSR and GC and possibly for synapse formation during CSR.

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DSBs induced by AID during CSR ultimately are joined by the efficient DNA repair pathway that requires C-NHEJ factors such as Ku70/80 (12, 23). However, in the absence of C-NHEJ, the A-EJ pathway that relies on microhomology can join the broken DNA ends, although this pathway is associated with chromosomal translocations (11, 24). Previously, we reported that JP8Bdel enhances aberrant c-myc/IgH translocations and that it fails to carry out the efficient recombination between donor and acceptor S regions in the IgH locus (8). Therefore, it is important to examine whether the AID C-terminal mutants affect the S–S joining in CSR.

In the current work we examined whether the C-terminal region of AID is involved in DNA synapse formation and recombination during CSR in CH12F3-2 and spleen B cells. We also examined the effect of AID C-terminal mutations on GC in chicken DT40 cells, which depends on HR between pseudo V genes and the downstream IgVλ region. Using these CSR- and GC-monitoring systems, we demonstrate that efficient CSR and GC require the C-terminal region of AID for the formation of DSB from SSB and subsequent end synapse. Considering these findings together, we conclude that, in addition to DNA cleavage, AID has a unique function in the generation of DSBs, which is required for S–S synapse formation and joining in CSR and recombination in GC.

Results and Discussion

AID C-Terminal Mutants Fail to Support NHEJ in CSR. Because the DNA cleavage in the Sμ region in AID C-terminal mutants remains unaltered during CSR (8), we examined whether the next step after DNA cleavage, namely recombination, is affected by these mutations. We analyzed junction microhomology of Sμ–Sγ1 recombination in AID-deficient splenic B cells retrovirally transfected with WT AID or P20 fused with GFP to monitor their expression (Fig. 1 *A* and *B*). Before transfection, cells were prestimulated with LPS for 2 d, and their IgG1 switching was induced by incubation with LPS and IL-4 for another 3 d. Consistent with the previous report, IgG1 switching was impaired in cells expressing P20 (Fig. 1*C*) (8, 16). DNA was isolated from IgG-1–switched cells and analyzed for the Sμ–Sγ1 junction. Interestingly, we found that the Sμ–Sγ1 junctions in P20-expressing cells contain less C-NHEJ repair signature (0–3 bp) and more frequent insertions, which presumably were introduced during repair by A-EJ, than do cells expressing WT AID (Fig. 1 *D* and *E*). The junctions with 0- to 3-bp microhomology occupied 59.2% and 28.9% in cells expressing WT AID and P20, respectively ($P < 0.05$). Our data showed longer average microhomology in the P20 transfectant: The average microhomology length was 5.65 bp for cells expressing P20 and 3.01 bp for cells expressing WT AID ($P < 0.05$).

We also examined the microhomology and insertions at the Sμ–Sα recombination in CH12F3-2 cells, which almost exclusively switch from IgM to IgA. CH12F3-2 cells expressing JP8Bdel-ER were activated by the addition of 4-hydroxytamoxifen (4-OHT) for 2 d, and IgA^+ cells were sorted to analyze their DNA for Sμ–Sα junction sequences. The phenotypes of the Sμ–Sα recombination junctions generated by JP8Bdel were essentially identical to those described above for the P20 mutation, with less C-NHEJ and more frequent insertions than in WT AID (Fig. 1 *F* and *G*). Our findings agree with the report of longer microhomology in the Sμ–Sα junctions in patients with the hyper-IgM syndrome type II ($AID^{+/C\text{-}term\Delta}$ carrying the AID C-terminal mutation R190X) than in healthy individuals (25). These results suggest that the C-terminal mutations of AID affect C-NHEJ drastically and affect A-EJ relatively less severely.

To confirm the NHEJ defect in AID C-terminal mutants, we examined the accumulation of bona fide repair proteins involved in C-NHEJ, such as Ku80 and XRCC4 in the Sμ region (23). SSBs were introduced by stimulating CH12F3-2 cells expressing WT AID-ER or JP8Bdel-ER with 1 μM 4-OHT for 3 h (8). Consistent with the microhomology analyses of the Sμ–Sγ1 and Sμ–Sα junction, our ChIP analysis showed a drastic reduction of Ku80 and XRCC4 in the Sμ region of CH12F3-2 cells expressing JP8Bdel-ER compared with those expressing WT AID-ER (Fig. 2*A*). These results indicate that the abolishment of CSR by P20 and

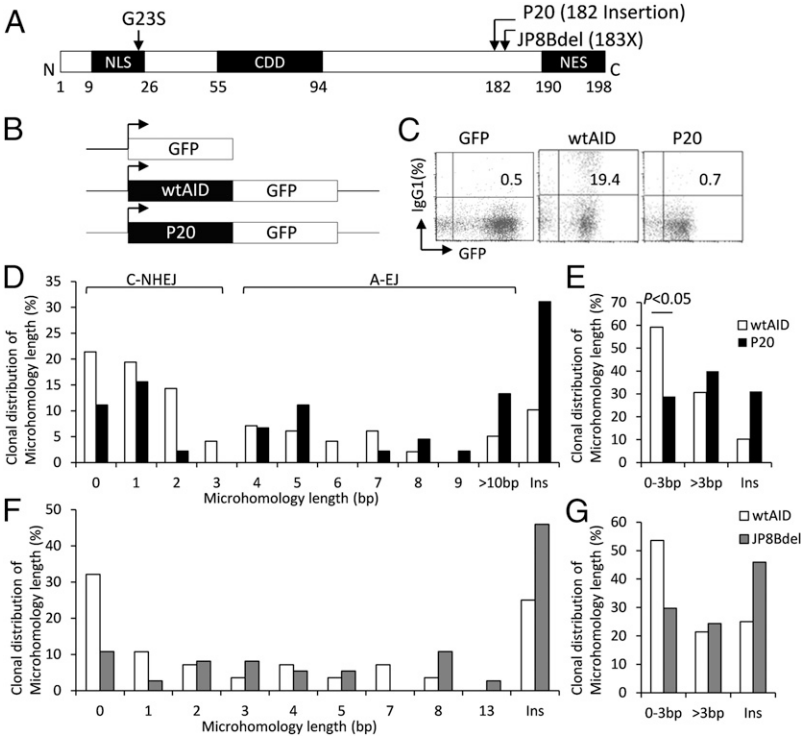

Fig. 1. Expression of AID C-terminal mutants suppresses C-NHEJ in CSR junctions. (*A*) Schematic representation of AID structure showing the locations of the nuclear localization signal (NLS), cytidine deaminase domain (CDD), and nuclear export signal (NES). Arrows indicate the N- and C-terminal mutants (G23S, P20, and JP8Bdel) with their respective mutations. (*B*) Schematic representation of retroviral expression constructs of GFP vector control and C-terminally GFP-fused WT AID and the P20 mutant. (*C*) FACS analysis of IgG1 switching in $AID^{-/-}$ spleen B cells following retroviral transduction of the indicated constructs and stimulation by LPS and IL-4 for 3 d. Percent CSR is indicated in each plot and was calculated based on GFP^+ $IgG1^+$ cells in the GFP gate. (*D* and *E*) Analysis of Sμ–Sγ1 junctions of DNA isolated from GFP^+IgG1^+ sorted cells following AID expression in AID-deficient spleen B cells as indicated. (*F* and *G*) Analysis of Sμ–Sα junctions of DNA isolated from IgA^+ sorted from CH12F3-2 cells expressing WT AID or JP8Bdel as indicated. *D* and *F* show C-NHEJ vs. A-EJ microhomology distributions. *E* and *G* plot average percent microhomology. Statistical significance was performed by Fisher's exact test.

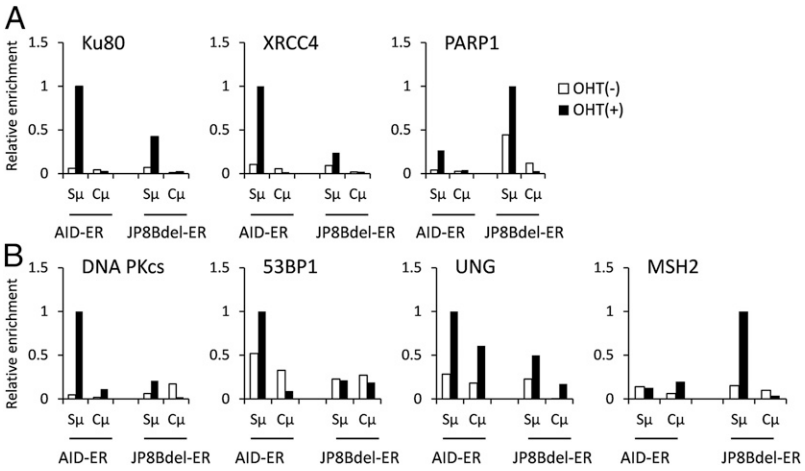

Fig. 2. WT but not the C-terminally defective AID mutant supports DNA end repair and synapse factor recruitment in the Sμ region. (*A*) ChIP assay of known C-NHEJ and A-EJ repair proteins in CH12F3-2 cells expressing either AID-ER or JP8Bdel-ER. (*B*) ChIP assay for S–S synapse-associated factors and the mismatch repair enzyme MSH2. Primers were specifically designed to examine the DNA break-prone Sμ region, and the Cμ region was selected as a DNA break-negative control zone. ChIP analysis was performed following AID activation by 4-OHT for 3 h. Rabbit IgG was used as a ChIP control antibody, and the background value was subtracted. Values were normalized to the DNA input. In each dataset, the maximum value is considered to be 1.0. Similar results were obtained in repeated independent experiments.

JP8Bdel mutants can be attributed to the impairment of end repairs and especially to the severe defect of the C-NHEJ pathway.

In contrast, we found the more abundant recruitment of an SSB-recognizing protein, poly (ADP)-ribose polymerase1 (PARP1), in the Sμ region in CH12 cells expressing JP8Bdel-ER (Fig. 2*A*). This result is consistent with the previous reports that PARP1 facilitates the A-EJ pathway (26) and preferentially binds to SSB (27, 28). Similarly, our ChIP data exhibited more abundant accumulation of MSH2 (Fig. 2*B*), a critical mismatch protein, at the Sμ region. This observation also is in agreement with the report that MSH2 plays an important role in the absence of C-NHEJ factors during CSR reaction (29). These data demonstrate that the AID C-terminal mutants fail to generate DSBs from SSBs and consequently fail to recruit proteins required for C-NHEJ. Collectively, the data show that the C-terminal mutants P20 and JP8Bdel are inefficient at the recombination step, as characterized by more severe defect in the C-NHEJ pathway.

AID C-Terminal Mutants Impair Sμ–Sα Synapse Formation. Because the defect in CSR of the C-terminal mutants of AID appears to affect the recombination step, we examined whether the interaction between Sμ and downstream S regions in the IgH locus also is affected. To this end, we examined the DNA synapse formation using the chromosome conformation capture (3C) assay in CH12F3-2 cells expressing WT AID-ER, G23S-ER (an N-terminal mutant), P20-ER, or JP8Bdel-ER. CSR was induced by 1 μM 4-OHT for 48 h. We first confirmed that CSR is compromised by the mutation at N-terminal region of AID and more severely by those mutations in C-terminal region, although their protein expression was not affected (Fig. 3 *A* and *B*). Subsequently, the stimulated cells were cross-linked in situ and subjected to a 3C assay (Fig. 3*C*), followed by restriction enzyme digestion and religation of the chromatin as described (*SI Materials and Methods*). The products were reverse cross-linked, and purified DNA was amplified by the specific primer pairs of two distant S regions (Fig. 3*D*). Although the Sμ–Sα interaction in the cells expressing endogenous AID was similar among all AID transfectants when stimulated with the CD40 ligand, IL4, and TGF-β (CIT), cells expressing the AID C-terminal mutants P20 and JP8Bdel showed significant reduction in Sμ–Sα synapse formation upon 4-OHT addition (without CIT stimulation) (Fig. 3*E*). On the other hand, the G23S mutation, which has defect in DNA cleavage but not in CSR (8, 9), demonstrated a level of Sμ–Sα interaction similar to that of WT AID. We sequenced the

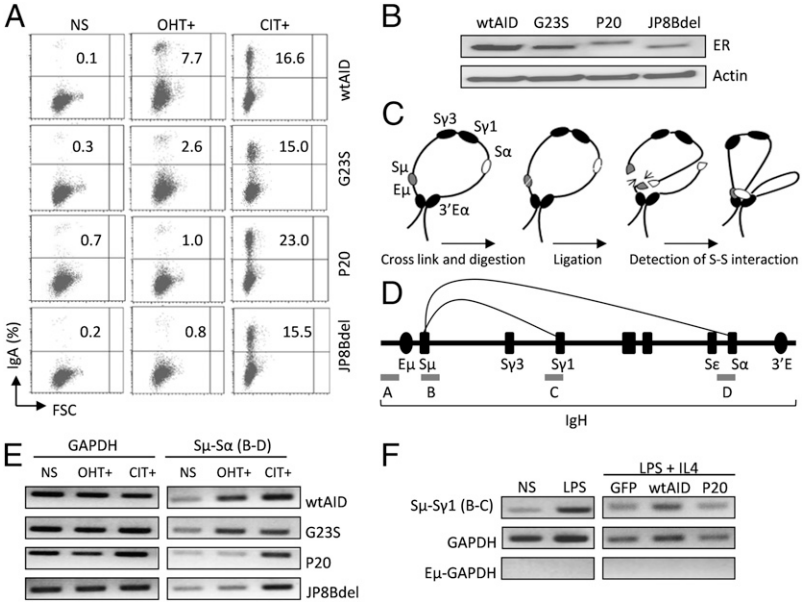

Fig. 3. Impaired Sμ–Sα/Sμ–Sγ1 synapse formation by AID C-terminal mutants. (*A*) FACS profiles of IgA switching in CH12 cells expressing ER-fused AID and its mutants as indicated. Cells were harvested after treatment with 1 μM 4-OHT for 48 h and stimulation by CIT for 24 h. The numbers in each panel indicate the percentage of IgA^+ cells. NS, cells not treated with either 4-OHT or CIT. (*B*) Examination of comparable expression levels of each AID construct in CH12F3-2 cells. AID expression and loading amount were monitored by anti-ER and anti-actin antibodies, respectively. (*C*) Schematic illustration of long-range interactions between Sμ–Sα elements in the gH locus that can be detected by the 3C method. (*D*) Schematic representation of long-range interactions examined in CH12F3-2 cells (Sμ–Sα, see *E*) stimulated by 4-OHT or CIT and in spleen B cells stimulated by LPS and IL-4 (Sμ–Sγ1, see *F*). The gray rectangles below the scheme indicate the primer positions in the 3C assay for Sμ–Sα and GAPDH control in CH12 cells expressing indicated constructs (*E*). Eμ-GAPDH did not show any product and was omitted. (*F*) Representative gel picture of the 3C assay in AID-deficient spleen B cells complemented by either WT AID or the P20 mutant. Treatment conditions are as follows: NS, nonstimulated (cells not treated with either 4- OHT or CIT); LPS, stimulated with LPS for 2 d; LPS + IL4, stimulated with LPS for 2 d followed by stimulation with LPS and IL-4 for 3 d.

amplified bands to confirm that amplified DNA fragments represent the joining of the two appropriate S regions by the designed 3C-PCR primer pairs (Fig. S1).

To confirm the defect in the long-range interaction in splenic B cells in the IgH locus, we expressed WT AID and P20 in AID-deficient spleen cells by retroviral infection and stimulated the infected cells with IL-4 and LPS for 3 d. WT AID or P20 was fused to GFP to monitor the level of protein expression in the infected cells. IgG1 switching by P20 was reduced drastically compared with WT AID in the GFP^+ populations (Fig. 1*C*). The 3C assay in spleen B cells confirmed the reduction of the Sμ–Sγ1 interaction in cells expressing P20 compared with cells expressing WT AID (Fig. 3*F*). The bands amplified by PCR were isolated and sequenced to exclude PCR artifacts (Fig. S1). These results clearly indicate that the long-range interactions in the IgH locus that form the S region synapse are severely impaired by the C-terminal mutants of AID. Taken together, these data demonstrate that the C-terminal region of AID is required for the formation of the S–S synapse during CSR. Wuerffel et al. (30) also reported the reduced levels of S–S synapsis in $AID^{-/-}$ spleen B cells. The authors speculated that the reduction of the interactions could be caused by the C-terminal scaffolding activity of the AID protein to mediate the long-range interaction in the IgH locus, although they did not suggest any molecular mechanism.

Reduction of the Critical DNA Synapse Factors in the Sμ Region by JP8Bdel. To confirm further the defect of synapse formation by AID C-terminal mutants, we tested the occupancy of the critical DNA synapse factors such as DNA PKcs and 53BP1 at the Sμ region (31–34). Using CH12F3-2 cells expressing JP8Bdel-ER, we found a drastic reduction of DNA synapse-forming proteins at the break sites compared with cells expressing WT AID (Fig. 2*B*). The results are consistent with the report that long-range microhomology is favored in the absence of 53BP1 and that 53BP1 protects DNA ends from resection (35, 36). We also examined the binding of UNG at the Sμ region in CH12F3-2 cells expressing JP8Bdel (Fig. 2*B*). In agreement with Ranjit et al. (37), our UNG ChIP data demonstrated that the accumulation of this protein is clearly AID dependent and that its accumulation is obviously reduced in cells expressing JP8Bdel compared with cells expressing WT AID. In contrast, the accumulation of the MSH2, an essential mismatch protein, was enhanced in the Sμ region. Taken together, these results indicate that the C-terminal mutants of AID are defective in recruiting critical synapse factors such as 53BP1, DNA PKcs, and UNG to the DSB site to support Sμ–Sα synapse formation.

C-Terminal Mutants of AID Reduced GC Efficiency. Because GC and CSR share similar mechanistic features, namely recombination, we next examined whether mutations in the C-terminal region of AID also affect HR during GC events. WT AID, P20, and JP8Bdel fused to ER were retrovirally introduced into $AID^{-/-}$ DT40 cells. The infected cells were selected with puromycin, and subcloning was performed. In this experiment, we used the cl18 DT40 chicken cell line, which carries a frameshift mutation in the complementary determining region 1 (CDR1) of the IgV_λ gene and expresses no surface IgM ($sIgM^-$). During GC, if this frameshift is repaired by HR with the pseudo V genes located upstream of the functional variable gene, the $sIgM^-$ DT40 cells revert to $sIgM^+$ cells (3). AID proteins were activated by 4-OHT for 4 wk, and GC was assessed by sIgM expression and IgV_λ region sequencing. We found that the sIgM level was reduced by more than threefold in P20-infected cells but was twofold higher in JP8Bdel-infected cells than in cells infected with WT AID (Fig. 4*A*). Western blot analysis in DT40 cells showed similar levels of AID expression among transfectants (Fig. 4*B*). The sIgM increment in JP8Bdel cells could be caused by the high rate of point mutations (PM) demonstrated for the S region (8).

To verify the sIgM reversion observed by flow cytometry, we cloned and sequenced the IgV_λ region from $sIgM^+$ cells. To enrich the $sIgM^+$ population in P20-infected cells, the reverted populations from cells stimulated by 4-OHT for 4 wk were singly or bulk sorted, and the IgV_λ region was analyzed (Fig. S2 and Table S1). As expected, the majority of the mutations were accumulated within CDR1 region Fig. S2). Interestingly, we observed that the GC percentage relative to PM is reduced in both P20-infected and JP8Bdel-infected cells compared with cells infected with WT AID, although this reduction is not as drastic as their abolishment of CSR (Fig. 4 *C* and *D* and Table S1). The ratios of PM combined with insertion/deletion (Ins/Del) events

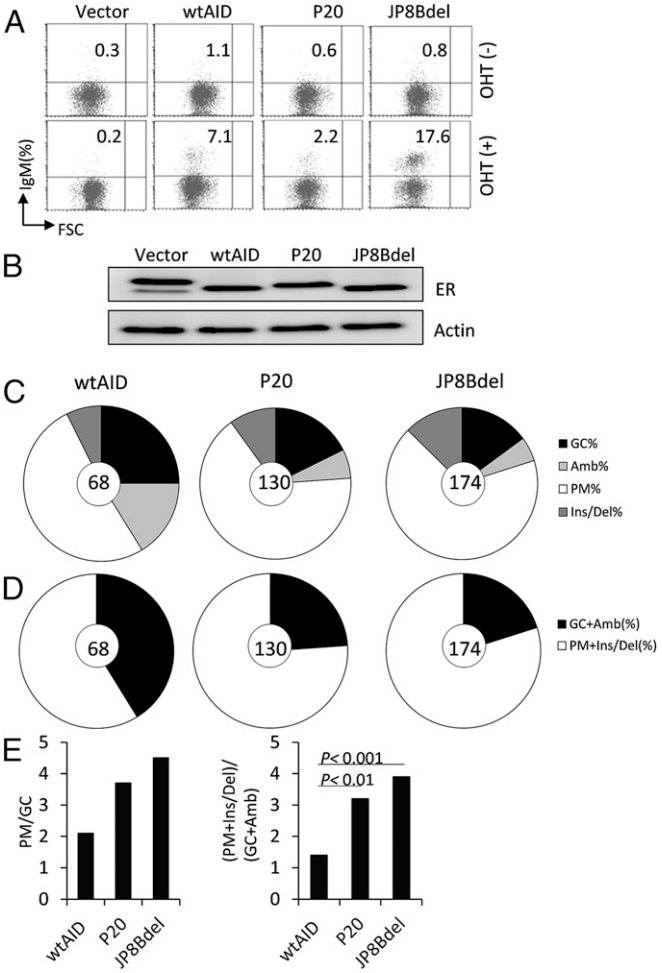

Fig. 4. Relative reduction of GC by AID C-terminal mutants in the chicken B-cell line DT40. (*A*) IgM reversion assay of retrovirally infected $AID^{-/-}$ DT40 cells with ER-fused WT AID or AID C-terminal mutant constructs. GFP-expressing retroviral vector was used as control. Numbers in the FACS profile indicate the percentage of reverted IgM cells treated with or without 1 μM 4-OHT after 4 wk. (*B*) Western blot showing the protein expression of DT40 transfectants with indicated constructs. Cells were harvested 3 d after puromycin selection. (*C*) IgVλ sequence analysis for the percentage of GC in $AID^{-/-}$ DT40 cells expressing the indicated AID C-terminal mutants. The pie charts show the relative distribution or proportion of GC, Amb, PM, and Ins/Del events. The total number of events is indicated in the center of each chart. (*D*) The pie charts show the proportion of events when GC and PM are combined with the Amb mutation and Ins/Del events, respectively. (*E*) (*Left*) Plot showing the proportion of PM to GC events. (*Right*) Combined proportion (%) of PM and Ins/Del vs. GC and Amb mutations in the sequenced IgVλ region. Data were collected from three independent subclones isolated for each AID mutant. Statistical significance was determined by Fisher's exact test.

to the GC combined with ambiguous mutations (Amb) were augmented significantly in mutants as compared with WT AID ($P < 0.01$ for WT AID vs. P20 and $P < 0.001$ for WT AID vs. JP8Bdel) (Fig. 4*E*). This partial defect in GC by C-terminal mutants of AID may be caused by the defect in generating DSB from SSB, because GC is known to be initiated by either DSB or SSB but probably is initiated more efficiently by DSB (38–40). Collectively, these data indicate that the C-terminal region of AID is required for the efficient processing of cleaved DNA ends to enable efficient recombination during CSR and GC.

Because we previously have shown that the CSR depends on de novo protein synthesis, we proposed that AID edits an mRNA to produce an unknown protein. We have shown that AID interacts with poly (A)-containing RNA at the C-terminal region of the AID protein (22). Considering our previous findings together with present results, we propose that mRNA edited by AID may encode an unknown factor that is essential for processing SSBs to DSBs that subsequently recruits synapse-forming proteins such as 53BP1, DNA PKcs, and UNG.

The Biochemical Step That Requires the Product of the C-Terminal Region of AID. It is tempting to speculate about the mechanism for generation of DSBs from AID-induced SSBs. We have provided a series of evidence that topoisomerase 1 (Top1) is responsible for generating SSBs immediately after AID activation (41, 42). Top1 generates SSBs only when it irreversibly forms a complex with the 3′ end of DNA. Top1 is known to associate covalently with non-B structure–forming DNA, which is abundant at repetitive sequences in the S region. For DNA repair, DNA-bound Top1 must be removed by proteasome, followed by TDP1/TPP digestion to expose the 3′ end of SSB (43–45). Because the S region is very rich in repetitive sequences, it can be assumed that SSB is generated on both strands of DNA within a reasonable distance that allows conversion of SSB to DSB by digestion by either exonuclease or endonuclease, extension by DNA polymerase, or unwinding by DNA helicase. There are several exonucleases and endonucleases that may be involved in CSR, including the MRN complex, Ape1, FEN1, and XPF-ERCC1. Because SHM is not affected by the C-terminal mutation of AID (7, 8, 16), it is unlikely to regulate the specific recruitment of any one of the enzymes that can convert SSB to DSB. Collectively, the AID C-terminal region product may affect all the enzymes involved in DSB processing.

The AID C-terminal product also may be involved in synapse formation after DSB formation. Consistently, DSBs generated by I-SceI (46) were not as efficient as those generated by AID, suggesting that AID may have another function in facilitating CSR. It is likely that the AID C-terminal product may share some functions with UNG, because both are required for efficient CSR and GC. However, UNG deficiency differs from the AID C-terminal mutation, having more severe phenotypes on GC (6), indicating that the two proteins have different roles after an AID-induced DNA break. Elucidation of the precise molecular function of the AID C-terminal production is critical for understanding the regulation of CSR.

Materials and Methods

Constructs, Transfection, and Retrovirus Infection. All AID proteins fused to GFP (used in spleen cell experiments) (17) or to the ER-puromycin–resistant gene (puro) (used in the DT40 experiment) have been described previously (8).

Cell Culture. Spleen B cells were obtained from 8- to 12-wk-old AID$^{-/-}$ mice with a C57/BL6 background. B cells pretreated with LPS were retrovirally infected with WT AID and P20-GFP and were stimulated with LPS and IL-4 for 3 d. The AID$^{-/-}$ DT40 cell line cl18 (47) was retrovirally infected by AID or various AID C-terminal mutants and was cultured with RPMI medium 1640 containing L-glutamine (Invitrogen), Penicillin-Streptomycin Mixed Solution (Nacalai Tesque) supplemented with 10% (vol/vol) FCS (Gibco) and 1% chicken serum (Gibco) at 39 °C. Twenty-four hours after infection, cells were treated with puromycin (0.5 μg/mL) for 3 d and then were applied in limited dilution in 96-well plates for 1 wk. The established single clones were stimulated with 50 nM 4-OHT and incubated for 4 wk. Cell medium was refreshed every 2 d.

Analysis of Sμ–Sγ1 and Sμ–Sα Junctions. The Sμ–Sγ1 region was amplified using high-fidelity PrimeSTAR DNA polymerase (TaKaRa) as previously described (48). After sorting of the switched cells and DNA extraction, nested PCR was performed. The primers used for the amplification of the Sμ–Sα region are listed in Table S2. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System and were cloned into pGEM-T Easy Vector (all from Promega). Sequencing was performed using T7 and SP6 primers, with an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

Flow Cytometry and Cell Sorting. Stimulated AID-deficient spleen B cells infected by WT AID or P20 were stained with biotinylated anti-mouse IgG1 (Becton Dickinson) followed by allophycocyanin-conjugated streptavidin (eBioscience). Anti-mouse IgA-phycoerythrin (Southern Biotech) was used for staining of IgA-switched CH12 cells. Dead cells were excluded by staining with propidium iodide. For analysis of the Sμ–Sγ1 junction in P20 cells, the IgG1^{+} population was enriched from stimulated cells by using magnetic beads (Anti-Mouse IgG1 Magnetic Particles-DM; BD IMag; BD Biosciences). All FACS analyses were carried out using FACSCalibur (Becton Dickinson). For analysis of the Sμ–Sα junction, IgA^{+} cells were sorted by FACSAria (BD Biosciences). Chicken IgM antibody (Bethyl) was labeled with the Alexa Fluor 647 kit (A20173) according to the provided protocol. After puromycin selection and limited dilution, subclones from all DT40 transfectants were stimulated by 4-OHT for 4 wk and were stained using chicken anti-IgM labeled with Alexa Floor 647. To enrich the IgM^{+} population in P20 cells for sequencing, bulk or single sorting was performed by FACSAria (BD Biosciences).

IgV$_\lambda$ GC and PM Analysis. After 4-OHT treatment for 4 wk, the P20 transfectant was sorted. The bulk-sorted IgM^{+} cells were cultured for 5 d before genomic DNA (gDNA) extraction, whereas gDNA from the single-sorted cells was extracted immediately and used subsequently for nested PCR. The IgV$_\lambda$ region was amplified and cloned according to the sorted method. For bulk-sorted cells, gDNA was extracted using phenol-chloroform protocol. The IgV$_\lambda$ region was amplified (47), cloned, and analyzed as described above. For single-sorted cells, DNA was extracted using Tween20 and proteinase K. The rearranged IgV$_\lambda$ fragments were amplified by nested PCR using 5′-GGTAT AAAAG GGCAT CGAGG TCCC -3′ (forward) and 5′-TAACC CTAAG TCCTC CATGG CGCA-3′ (reverse) as an outer primer (35 cycles). The inner primer was the primer used for IgV$_\lambda$ amplification in bulk-sorted cells (47). The PCR product was purified by the Exo-SAP-IT kit (78200; Affymetrix) and was applied directly for sequencing as described above. All modifications within entire rearranged IgV$_\lambda$ fragments were categorized into four groups: GC, Amb mutation, PM, or Ins/Del. This classification is based on the accessibility of the pseudo V donor genes, searched in the database http://blast.ddbj.nig.ac.jp/blast/blastn?lang=ja3, which could be considered as a template. A mutation tract harboring a >9-bp string homologous to the pseudo V donor was considered a GC. If there was only one hit in the sequence, and such a mutation was found in database, it was categorized as an Amb mutation. A mutation that was not found in donor pseudo genes was identified as a PM. In this categorization, any additional or deleted base along the IgV$_\lambda$ region was considered an Ins or Del, respectively.

ChIP. We used the Active Motif ChIP-IT Express Kit according to the manufacturer's instructions with slight modifications as reported elsewhere (49). Briefly, 5×10^6CH12 AID/JP8Bdel ER cells were fixed for 5 min in the presence of 1% formaldehyde at room temperature. To quench the reaction, glycine to a final concentration of 0.125 M was added with rotation for 5 min. After the cross-linking reaction was washed, cell lysis and sonication were performed. The sheared samples were subjected to reverse cross-linking and were treated with proteinase K and RNaseA. Following electrophoresis, the sheared chromatin that yielded a smear between 200 and 1,000 bp was used for immunoprecipitation. Antibody (2–3 μg) was added to the lysate with rotation at 4 °C overnight. After the beads were washed, the immunoprecipitated DNA was used for real-time PCR using SYBR Green Master Mix (Applied Biosystems). The quantitative PCR signal was normalized by the input. The dataset was recalculated and the maximum value was considered as 1.0. Primers and antibodies used are listed in Tables S2 and S3, respectively.

3C Assay. The 3C assay was performed as described previously (30), with minor modifications in CH12 cells. Additional details of the 3C assay are given in *SI Materials and Methods*.

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1. Muramatsu M, et al. (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102(5):553–563.
2. Yoshikawa K, et al. (2002) AID enzyme-induced hypermutation in an actively transcribed gene in fibroblasts. *Science* 296(5575):2033–2036.
3. Arakawa H, Hauschild J, Buerstedde JM (2002) Requirement of the activation-induced deaminase (AID) gene for immunoglobulin gene conversion. *Science* 295(5558): 1301–1306.
4. Revy P, et al. (2000) Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* 102(5):565–575.
5. Okazaki IM, Kinoshita K, Muramatsu M, Yoshikawa K, Honjo T (2002) The AID enzyme induces class switch recombination in fibroblasts. *Nature* 416(6878):340–345.
6. Saribasak H, et al. (2006) Uracil DNA glycosylase disruption blocks Ig gene conversion and induces transition mutations. *J Immunol* 176(1):365–371.
7. Barreto V, Reina-San-Martin B, Ramiro AR, McBride KM, Nussenzweig MC (2003) C-terminal deletion of AID uncouples class switch recombination from somatic hypermutation and gene conversion. *Mol Cell* 12(2):501–508.
8. Doi T, et al. (2009) The C-terminal region of activation-induced cytidine deaminase is responsible for a recombination function other than DNA cleavage in class switch recombination. *Proc Natl Acad Sci USA* 106(8):2758–2763.
9. Shinkura R, et al. (2004) Separate domains of AID are required for somatic hypermutation and class-switch recombination. *Nat Immunol* 5(7):707–712.
10. Petersen S, et al. (2001) AID is required to initiate Nbs1/gamma-H2AX focus formation and mutations at sites of class switching. *Nature* 414(6864):660–665.
11. Boboila C, et al. (2010) Alternative end-joining catalyzes robust IgH locus deletions and translocations in the combined absence of ligase 4 and Ku70. *Proc Natl Acad Sci USA* 107(7):3034–3039.
12. Yan CT, et al. (2007) IgH class switching and translocations use a robust non-classical end-joining pathway. *Nature* 449(7161):478–482.
13. Lieber MR (2010) NHEJ and its backup pathways in chromosomal translocations. *Nat Struct Mol Biol* 17(4):393–395.
14. Lieber MR (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem* 79:181–211.
15. Sale JE, Calandrini DM, Takata M, Takeda S, Neuberger MS (2001) Ablation of XRCC2/3 transforms immunoglobulin V gene conversion into somatic hypermutation. *Nature* 412(6850):921–926.
16. Ta VT, et al. (2003) AID mutant analyses indicate requirement for class-switch-specific cofactors. *Nat Immunol* 4(9):843–848.
17. Ito S, et al. (2004) Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. *Proc Natl Acad Sci USA* 101(7):1975–1980.
18. Shivarov V, Shinkura R, Honjo T (2008) Dissociation of in vitro DNA deamination activity and physiological functions of AID mutants. *Proc Natl Acad Sci USA* 105(41): 15866–15871.
19. Honjo T, et al. (2012) The AID dilemma: Infection, or cancer? *Adv Cancer Res* 113:1–44.
20. Geisberger R, Rada C, Neuberger MS (2009) The stability of AID and its function in class-switching are critically sensitive to the identity of its nuclear-export sequence. *Proc Natl Acad Sci USA* 106(16):6736–6741.
21. Ellyard JI, Benk AS, Taylor B, Rada C, Neuberger MS (2011) The dependence of Ig class-switching on the nuclear export sequence of AID likely reflects interaction with factors additional to Crm1 exportin. *Eur J Immunol* 41(2):485–490.
22. Nonaka T, et al. (2009) Carboxy-terminal domain of AID required for its mRNA complex formation in vivo. *Proc Natl Acad Sci USA* 106(8):2747–2751.
23. Boboila C, Alt FW, Schwer B (2012) Classical and alternative end-joining pathways for repair of lymphocyte-specific and general DNA double-strand breaks. *Adv Immunol* 116:1–49.
24. Ferguson DO, et al. (2000) The nonhomologous end-joining pathway of DNA repair is required for genomic stability and the suppression of translocations. *Proc Natl Acad Sci USA* 97(12):6630–6633.
25. Kracker S, et al. (2010) Impaired induction of DNA lesions during immunoglobulin class-switch recombination in humans influences end-joining repair. *Proc Natl Acad Sci USA* 107(51):22225–22230.
26. Robert I, Dantzer F, Reina-San-Martin B (2009) Parp1 facilitates alternative NHEJ, whereas Parp2 suppresses IgH/c-myc translocations during immunoglobulin class switch recombination. *J Exp Med* 206(5):1047–1056.
27. Dantzer F, et al. (2000) Base excision repair is impaired in mammalian cells lacking Poly(ADP-ribose) polymerase-1. *Biochemistry* 39(25):7559–7569.
28. Molinete M, et al. (1993) Overproduction of the poly(ADP-ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells. *EMBO J* 12(5):2109–2117.
29. Eccleston J, Yan C, Yuan K, Alt FW, Selsing E (2011) Mismatch repair proteins MSH2, MLH1, and EXO1 are important for class-switch recombination events occurring in B cells that lack nonhomologous end joining. *J Immunol* 186(4):2336–2343.
30. Wuerffel R, et al. (2007) S-S synapsis during class switch recombination is promoted by distantly located transcriptional elements and activation-induced deaminase. *Immunity* 27(5):711–722.
31. DeFazio LG, Stansel RM, Griffith JD, Chu G (2002) Synapsis of DNA ends by DNA-dependent protein kinase. *EMBO J* 21(12):3192–3200.
32. Chapman JR, et al. (2013) RIF1 is essential for 53BP1-dependent nonhomologous end joining and suppression of DNA double-strand break resection. *Mol Cell* 49(5): 858–871.
33. Di Virgilio M, et al. (2013) Rif1 prevents resection of DNA breaks and promotes immunoglobulin class switching. *Science* 339(6120):711–715.
34. Zimmermann M, Lottersberger F, Buonomo SB, Sfeir A, de Lange T (2013) 53BP1 regulates DSB repair using Rif1 to control 5′ end resection. *Science* 339(6120): 700–704.
35. Bothmer A, et al. (2010) 53BP1 regulates DNA resection and the choice between classical and alternative end joining during class switch recombination. *J Exp Med* 207(4):855–865.
36. Difilippantonio S, et al. (2008) 53BP1 facilitates long-range DNA end-joining during V(D)J recombination. *Nature* 456(7221):529–533.
37. Ranjit S, et al. (2011) AID binds cooperatively with UNG and Msh2-Msh6 to Ig switch regions dependent upon the AID C terminus. *J Immunol* 187(5):2464–2475.
38. Nakahara M, et al. (2009) Genetic evidence for single-strand lesions initiating Nbs1-dependent homologous recombination in diversification of Ig v in chicken B lymphocytes. *PLoS Genet* 5(1):e1000356.
39. Tang ES, Martin A (2006) NHEJ-deficient DT40 cells have increased levels of immunoglobulin gene conversion: Evidence for a double strand break intermediate. *Nucleic Acids Res* 34(21):6345–6351.
40. Cook AJ, et al. (2007) DNA-dependent protein kinase inhibits AID-induced antibody gene conversion. *PLoS Biol* 5(4):e80.
41. Kobayashi M, et al. (2009) AID-induced decrease in topoisomerase 1 induces DNA structural alteration and DNA cleavage for class switch recombination. *Proc Natl Acad Sci USA* 106(52):22375–22380.
42. Kobayashi M, et al. (2011) Decrease in topoisomerase I is responsible for activation-induced cytidine deaminase (AID)-dependent somatic hypermutation. *Proc Natl Acad Sci USA* 108(48):19305–19310.
43. Pommier Y, et al. (2003) Repair of and checkpoint response to topoisomerase I-mediated DNA damage. *Mutat Res* 532(1-2):173–203.
44. Zhang YW, et al. (2011) Poly(ADP-ribose) polymerase and XPF-ERCC1 participate in distinct pathways for the repair of topoisomerase I-induced DNA damage in mammalian cells. *Nucleic Acids Res* 39(9):3607–3620.
45. Lin CP, Ban Y, Lyu YL, Liu LF (2009) Proteasome-dependent processing of topoisomerase I-DNA adducts into DNA double strand breaks at arrested replication forks. *J Biol Chem* 284(41):28084–28092.
46. Zarrin AA, et al. (2007) Antibody class switching mediated by yeast endonuclease-generated DNA breaks. *Science* 315(5810):377–381.
47. Hirota K, et al. (2010) Simultaneous disruption of two DNA polymerases, Polη and Polζ, in Avian DT40 cells unmasks the role of Polη in cellular response to various DNA lesions. *PLoS Genet* 6(10):e1001151.
48. Sabouri Z, et al. (2009) Apex2 is required for efficient somatic hypermutation but not for class switch recombination of immunoglobulin genes. *Int Immunol* 21(8):947–955.
49. Stanlie A, Aida M, Muramatsu M, Honjo T, Begum NA (2010) Histone3 lysine4 trimethylation regulated by the facilitates chromatin transcription complex is critical for DNA cleavage in class switch recombination. *Proc Natl Acad Sci USA* 107(51): 22190–22195.